Isolation and identification of a novel cellular protein that potently activates Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C)

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A novel heat-stable cellular protein that significantly stimulated Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C, PKC) was identified. It is ubiquitous and has a molecular mass of over 150 kDa, and was shown to be specific for PKC. It activates PKC in the absence and presence of phospholipids; however, its maximal stimulatory potential was achieved when optimal amounts of phospholipids were also present in the reaction medium. Thus, in comparison with classical cofactors such as phospholipid, where activation of PKC mainly involves interaction with the regulatory domain of PKC, the mechanism of activation of PKC by the activator appears to involve several binding sites.

INTRODUCTION

Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C, PKC) represents a multigene family consisting of various enzyme subspecies [1–4]. PKC functions as a ubiquitous cellular mediator of signal transduction initiated by a wide variety of agonists, and is the receptor for phorbol ester and other tumour promoters [5]. With certain substrates such as histones, the phosphorylation catalysed by these enzymes is known to be regulated by several cofactors, such as phospholipids, phorbol esters (or diacylglycerols) and Ca^{2+} [6]. These cofactors activate the enzyme by interacting with the autoinhibitory pseudo-substrate region in the regulatory domain of the enzyme [7]. It is also known that the catalytic fragment of PKC (PKM), generated by trypsin treatment of the enzyme, catalyses phosphorylation of protein substrates in a cofactor-independent manner [2]. Thus the inhibition of the kinase activity exerted by the pseudo-substrate can be alleviated by addition of the cofactors or via removal of the regulatory domain (containing the pseudo-substrate domain) by trypsin treatment of the enzyme to generate PKM.

In previous studies, I have shown that PKC isoenzymes in the rat ventral prostate are differentially regulated by androgens [8]. Several factors may be considered to account for such findings, i.e. a change in transcription or post-transcription of the various isoenzyme forms and/or alteration in their stability, or a change in the level of activators or inhibitors of these kinases. A naturally occurring small-molecular-mass protein which inhibited PKC has been identified, and its amino acid sequence was recently reported [9]. I have searched for modulators of PKC activity that may have caused the changes in prostatic PKC activities that I observed in previous studies [8]. Here, I report on the identification of a naturally occurring activator of PKC. In addition to the prostate, the activator was also present in other tissues investigated, such as brain. It enhanced the PKC-catalysed phosphorylation of histones by several-fold. The activator increased the PKC activity in the absence and presence of the various cofactors, indicating that the activator may also act at or

near the active site of the enzyme. This is the first report on the identification of a naturally occurring cellular protein which acts as a potent activator of PKC.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats weighing 295–325 g, purchased from Harlan Sprague–Dawley Co., Indianapolis, IN, U.S.A., were used to isolate various tissues used in this study. They were maintained as described previously [8].

Chemicals

Lysine-rich histone H1 and DNAase I were purchased from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Diolein (DO), phosphatidylserine (PS), phenylmethanesulphonyl fluoride (PMSF), leupeptin, RNAase and chymotrypsin were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. DEAE-cellulose DE-52 (DE-52) was purchased from Whatman LabSales, Hillsboro, OR, U.S.A. Other reagents were of the highest purity available.

Determination of PKC activity

This was done in a 0.15 ml reaction mixture consisting of 10 mM- MgCl_2 , 0.5 mM- CaCl_2 , 100 μg of PS/ml, 10 μg of DO/ml, 0.1 mM- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (sp. radioactivity 100–500 d.p.m./pmol of ATP), 20 mM-Tris/HCl, pH 7.5, and 1 mg of lysine-rich histone H1/ml. The reaction was started by addition of the enzyme diluted in buffer A (20 mM-Tris/HCl, pH 7.5, 10 mM-2-mercaptoethanol) containing 100 μg of ovalbumin/ml, and was carried out for 0, 3 and 6 min at 37 °C. PKC activity was determined as nmol of ^{32}P incorporated in the substrate/h per mg of enzyme protein in the presence of Ca^{2+} /phospholipids minus that in the absence of Ca^{2+} /phospholipids [8]. When stimulation of the enzyme activity by the activator was investigated, PKC activity was determined in the presence of a portion of buffer containing the activator and compared with that assayed in the presence of

Abbreviations used: PKC, protein kinase C (Ca^{2+} /phospholipid-dependent protein kinase); CK-I, cytosolic casein kinase I; CK-II, cytosolic casein kinase II; NK-I, nuclear casein kinase I; NK-II, nuclear casein kinase II; PKA, protein kinase A (cyclic AMP-dependent protein kinase); PS, phosphatidylserine; DO, diolein; PMSF, phenylmethanesulphonyl fluoride.

an equal amount of control protein (ovalbumin) in the presence and absence of Ca^{2+} /phospholipids.

Determination of enzymic activity of other protein kinases

Cytosolic casein kinase I (CK-I), cytosolic casein kinase II (CK-II), nuclear casein kinase I (NK-I) and nuclear casein kinase II (NK-II) were purified from rat liver as described previously [10,11]. Bovine heart cyclic AMP-dependent protein kinase Type II (protein kinase A, PKA) was purchased from Sigma Chemical Co. Protein kinase activities of these enzymes were determined with casein as substrate for the casein kinases, and with lysine-rich histone H1 in the presence of 5 μM -cyclic AMP for PKA [12].

Partial purification of the activator

This was carried out with rat brain, since it was found to be a rich source of the activator. Briefly, 10 rat brains were homogenized and the supernatant and pellet fractions were prepared as previously described [8]. Both fractions were pooled and heated for 5 min at 95 °C, cooled on ice, and centrifuged at 20000 g (r_{av} 6.98 cm) for 5 min. The supernatant was applied to a 5 ml DE-52 DEAE-cellulose column equilibrated in buffer A. The column was washed with the same buffer, and the activator was recovered by eluting the column with buffer A containing 0.2 M-NaCl. The eluate was concentrated and applied to a Sephacryl S-200 column which was previously equilibrated in buffer A containing 300 mM-NaCl. A 50 μl sample from each fraction was tested for the presence of the activator, and fractions which were found to increase PKC activity by 2-fold over control were pooled, concentrated and used for the studies shown below.

Other procedures

PKC was purified from brain by a protocol that I have described previously [8], but an additional step involving a phenyl-Sepharose column was included [13]. Protein was determined by a dye-binding assay with BSA as the standard [14]. Enzyme digestion of the activator was carried out in the presence of 5 μg of DNAase I, RNAase A or chymotrypsin per 50 μg of activator under optimal reaction conditions for these enzymes and incubated for 3 h at 37 °C, followed by heat inactivation for 10 min at 100 °C for DNAase or chymotrypsin, or by addition of EDTA to 0.5 mM for RNAase A.

RESULTS AND DISCUSSION

Isolation of the activator

The isolation and partial purification of the activator were carried out with rat brain, since it was found to be a rich source for this activator (results not shown). Briefly, the activator was shown to be heat-stable, and therefore we took advantage of this property in the purification protocol. By heating the brain extract to 95 °C for 5 min, we were able to remove more than 80 % of the total protein, which was discarded by centrifugation, and thus a smaller-size DE-52 column was sufficient for purifying the activator. From 10 rat brains we obtained about 250 mg of protein in the cytosol + pellet pool, and by heating this pool we were able to remove over 240 mg of protein. The remaining 5–7 mg was applied to the DE-52 column, and the activator was eluted with 200 mM-NaCl. The activator-containing fractions were pooled, concentrated, and applied to the Sephacryl S-200 column. The activator appeared early in the column profile, which indicates a high apparent molecular mass (over 150 kDa). The amount of activator recovered in the Sephacryl S-200 pool was about 200 μg of protein. We have employed the Sephacryl-S-200-concentrated pool as the source of the activator for the studies presented below.

Studies on the mechanism of activation of PKC by the activator

Although most of the studies on the effect of the activator on PKC were carried out with purified type II (β) isoenzyme, similar results were obtained when type III (α) was investigated. Therefore, I present here the data obtained with type II isoenzyme. The results in Table 1 show that a remarkable stimulation of PKC was observed on addition of the activator. At a very low level of the activator (5 μg), there was a 2-fold stimulation of PKC activity. Further stimulation of the kinase activity was observed with higher amounts of the activator, i.e. 4- and 7-fold increase in activity with 12.5 and 50 μg of activator respectively. No additional increase in stimulation of PKC activity was observed in the presence of higher concentrations of activator. Thus saturable binding kinetics exist between the enzyme and the activator; however, the exact stoichiometry cannot be determined at present unless the activator is purified to homogeneity.

Since enzymes are generally found to be unstable when present in very dilute form, and can be stabilized by addition of proteins such as ovalbumin or BSA in the reaction, we have investigated whether the activation of PKC was the result of a stabilization of the enzyme by the proteins contributed by adding the activator. Therefore the effect of protein concentration on the total enzyme activity, as well as on the fold stimulation of the kinase activity by the activator, was studied. To this end, we carried out experiments in the presence of various amounts of ovalbumin, and in the presence and absence of the activator. As expected, the addition of 100 μg of ovalbumin/ml resulted in an increase in kinase activity by 2-fold, and no further increase in the activity was observed at higher concentrations of ovalbumin (1 mg/ml) (Table 2). The addition of 10 μg of activator per reaction mixture (final concn. 66 $\mu\text{g}/\text{ml}$) resulted in stimulation of PKC activity

Table 1. Effect of various amounts of the partially purified activator on PKC activity

PKC activity was determined in the presence of 100 μg of PS/ml, 10 μg of DO/ml, 0.5 mM- CaCl_2 , and in the absence or presence of various amounts of activator. Results are expressed as means $\pm$ S.D. ($n = 12$).

Activator (μg of protein)	PKC activity (nmol of ^{32}P /h per mg)	Stimulation (fold)
None	8960 $\pm$ 80	1.00
5.0	18850 $\pm$ 110	2.10
12.5	36210 $\pm$ 120	4.10
50.0	60100 $\pm$ 150	6.70
125.0	65190 $\pm$ 150	7.30

Table 2. Effect of protein concentration on the effectiveness of the activator in stimulating PKC activity

PKC activity was determined in the presence of 100 μg of PS/ml, 10 μg of DO/ml, 0.5 mM- CaCl_2 and 10 μg of activator/ml, in the presence of various amounts of ovalbumin. Results are expressed as means $\pm$ S.D. ($n = 6$).

Ovalbumin concn. (mg/ml)	PKC activity (nmol of ^{32}P /h per mg)		Stimulation (fold)
	No activator	Plus activator	
0	7680 $\pm$ 70	28800 $\pm$ 100	3.8
0.1	13680 $\pm$ 90	52800 $\pm$ 150	3.86
1.0	12960 $\pm$ 90	46500 $\pm$ 120	3.6

Table 3. PKC activity in the presence and absence of activator and histone H1

Enzyme was assayed in the presence of 100 μ g of PS/ml, 10 μ g of DO/ml and 0.5 mM-CaCl₂. Histone H1 and activator were present at 1 mg/ml and 50 μ g/ml respectively. Results are expressed as means $\pm$ S.D. ($n = 6$).

Addition	PKC activity (nmol of ³² P/h per mg)
PKC + H1 + activator	45000 $\pm$ 120
PKC + H1	9500 $\pm$ 24
PKC + activator	1250 $\pm$ 15

Table 4. Effect of addition of the activator on enzymic activity of various protein kinases

Protein kinase activities were determined as described previously [12] in the absence or presence of 50 μ g of activator per reaction. Results are expressed as means $\pm$ S.D. ($n = 6$).

Enzyme	Protein kinase activity (μ mol of ³² P/h per mg)	
	No activator	Plus activator
PKA	9 $\pm$ 2.0	12 $\pm$ 3.0
CK-I	45 $\pm$ 1.3	45 $\pm$ 1.2
NK-I	50 $\pm$ 1.3	50 $\pm$ 1.3
CK-II	80 $\pm$ 2.5	80 $\pm$ 2.5
NK-II	70 $\pm$ 2.4	70 $\pm$ 2.4

by about 4-fold in the presence and absence of ovalbumin. However, the absolute values for PKC activity were about 2-fold higher in the presence of ovalbumin. Thus, although the total activity of the kinase was higher in the presence of ovalbumin, owing to an increase in enzyme stability, the fold stimulation by the activator was independent of protein concentration. It was also essential to establish that the remarkable increase in ³²P incorporation was not related to the fact that the activator served merely as a substrate for PKC. The PKC-catalysed phosphorylation was carried out in the absence and presence of activator, and in the absence and presence of histone H1. The results in Table 3 show that activator addition to the enzyme did not significantly enhance ³²P incorporation compared with that incorporated in H1. Thus it appears that the activator is not a good substrate for PKC, and that the activator is required for the stimulation of PKC-catalysed phosphorylation of H1.

To check whether the stimulation by the activator was specific for PKC, the effect of addition of the activator on the kinase activity of various protein kinases was also investigated. The enzymes tested in our system were CK-I, CK-II, NK-I and NK-II and PKA. The activator, added at the concentration which gave about 6–7-fold stimulation of PKC, did not alter the casein kinase activity of CK-I, CK-II, NK-I and NK-II, and only about 30% stimulation of histone phosphorylation by PKA was observed in the presence of the activator (Table 4). Thus it appears that the stimulatory effect is specific to PKC, but further investigations of the effect of the activator on the kinase activity of other protein kinases are warranted.

In order to determine the chemical nature of this activator, we subjected the Sephacryl S-200 pool to various nucleolytic and proteolytic enzyme treatments as described in the Materials and

Table 5. Effect of various enzyme treatments of the partially purified activator on its effectiveness in stimulating PKC activity

A 15 μ g sample of activator was treated with buffer or with various enzymes before being tested for stimulation of PKC activity under optimal PKC assay conditions (100 μ g of PS/ml, 10 μ g of DO/ml, 0.5 mM-CaCl₂). Results are expressed as means $\pm$ S.D. ($n = 6$).

Treatment	PKC activity	
	(nmol of ³² P/h per mg)	(% of control)
No activator	11 560 $\pm$ 80	100
Activator and:		
Buffer	74 550 $\pm$ 120	647
RNAase	75 500 $\pm$ 110	652
DNAase	75 680 $\pm$ 124	656
Chymotrypsin	9775 $\pm$ 125	85

Table 6. Effect of various concentrations of PS on the kinase activity of PKC in the absence and presence of the activator

PKC activity was determined in the presence of 10 μ g of DO/ml, 0.5 mM-CaCl₂, and various amounts of PS and in the absence or presence of 15 μ g of activator. Results are expressed as means $\pm$ S.D. ($n = 6$).

PS concn. (μ g/ml)	PKC activity (nmol of ³² P/h per mg)		Stimulation (fold)
	No activator	Plus activator	
None	1260 $\pm$ 15	7050 $\pm$ 80	5.60
20	7740 $\pm$ 80	36480 $\pm$ 120	4.70
40	8670 $\pm$ 80	43320 $\pm$ 150	5.00
60	8670 $\pm$ 85	49020 $\pm$ 155	5.65
80	8670 $\pm$ 85	49020 $\pm$ 140	5.65

methods section. The results shown in Table 5 indicate that the stimulation of the kinase activity by the activator was not affected by DNAase I or RNAase A treatment of the activator. Addition of chymotrypsin to the activator resulted in loss of its stimulatory effect on the kinase activity. The possibility that the stimulation of PKC was due to a proteolytic activity of the activator was ruled out, since the electrophoretic profile of PKC was similar to that of the enzyme which had been preincubated with the activator, i.e. no degradation of PKC was observed. Thus it appears that the activator is a high-molecular-mass protein molecule, is not a substrate for PKC, is not a protease, and it is a potent stimulator for PKC in the absence and presence of effector molecules. The exact identity and physical characteristics of this macromolecule will require further purification to homogeneity.

Since phospholipids are known to activate PKC by interacting with the inhibitory region on the regulatory domain of the enzyme [7], and since mutagenesis of the pseudo-substrate site of PKC results in an effector-independent constitutively active enzyme [15], it was decided to examine the mechanism by which the activator stimulates PKC. PKC activity was determined in the presence of various concentrations of PS, and in the presence and absence of the activator. It appears that the activator stimulated the kinase activity in the absence of PS to the same level observed in their presence without the activator (Table 6). However, the addition of increasing amounts of PS in the presence of the activator resulted in further enhancement of kinase activity. Thus, although the activator can activate the

enzyme in the absence of PS, the addition of increasing amounts of PS in the presence of the activator resulted in further enhancement of the kinase activity. Other cofactors such as Ca^{2+} and DO were also investigated, and no effect of such factors on the fold stimulation of PKC by the activator was observed, i.e. the activation by the activator was independent of their concentration. The activation by this stimulator is different from the classical mechanism of PKC activation by phospholipids. It is well documented that lipid-dependent activation of PKC by phospholipids results from the interaction of these lipids with the regulatory domain, thus removing the inhibitory effect of the pseudo-substrate [1–4]. The results presented here show that, although the activator may bind directly to the catalytic domain of the enzyme and produce an increase in its catalytic activity, optimal stimulatory potential of the enzyme by the activator requires the presence of PS, which may indicate that additional binding sites may be involved. Also, the mechanism of activation by this activator appears to be different from that we observed with heparin [16]. We have shown that heparin stimulated PKC activity by interacting with the regulatory domain, and lowering the requirement for PS. Thus, although heparin can mimic PS in stimulating PKC activity in the absence of PS, this stimulation by heparin is decreased or even eliminated when an optimal concentration of PS was present. In comparison with heparin, the fold stimulation by the naturally occurring activator is not minimized or affected by PS in relative terms, although maximal total activity of PKC is achieved in the presence of both PS and the activator. Finally, our results show that this is the first activator to interact with the catalytic domain of PKC, and also it is the first macromolecule to be isolated that potentially activates this enzyme. The purification of the activator to homogeneity

will pave the way for additional studies of the precise mechanism of activation of the PKC by this activator.

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